HISTOCHEMICAL AND BIOCHEMICAL OBSERVATIONS ON THE ALDOSTERONE-

STIMULATED URINARY BLADDER OF THE TOAD, BUFO MARINUS

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Δίδοτε καὶ δοθήσεται ὑμῖν· Μέτρον καλὸν, πεπιεσμένον καὶ σεσαλευμένον καὶ υπερεκχυνόμενον δώσουσιν εἰς τὸν κόλπον ὑμῶν·

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TABLE OF CONTENTS

INTRODUCTION

A. The toad urinary bladder and sodium transport.

The toad Bufo marinus is a ubiquitous amphibian inhabitant of the
Colombian Coast of South America where it is found in brackish marsh-
land (Gadow, '01). Among the many adaptions to terrestial life mani-
fested by the toad, the urinary bladder has long been thought to play
a prominent role in the animal's ecology as a fluid reservoir (Steen, '29).
The urinary bladder reaches its largest size among the Amphibia in cer-
tain species of Bufo (Noble, '31). Leaf ('55) discovered that the
urinary bladder possessed the capacity to transport sodium ions in the
same manner as frog skin in the model system of Ussing and Zerahn. The
latter workers (Ussing and Zerahn, '51) demonstrated that the endogenous
electrical activity of the frog skin was proportional to the amount of
sodium transported across the membrane, and thus ion transport could be
studied electrically with the so-called "short circuit technique" in
which an epithelial membrane is positioned between two chambers containing
an electrolyte solution in which current can be measured. The simpler
structure of the urinary bladder, with its absence of glands and thick
connective tissue stroma, is even more suitable for this purpose than the
frog integument.

Primarily from experiments designed after the Ussing-Zerahn model,
a theory of ionic transport for the toad urinary bladder has evolved

which is felt to be applicable to a certain extent to other tissues
engaged in active transport as well (see Sharp and Leaf, '66). Sodium
ions are thought to move passively from the urine into the mucosal cell
where by means of the "sodium pump" the ion is transported across an
electrical gradient to the extracellular compartment of the animal
generating an electrical potential. The process is dependent upon
cellular metabolism (Leaf et al., '59) and is inhibited by cardiac glyco-
sides. Leaf et al. ('58) have shown that the urinary bladder provides
an effective reabsorption mechanism since sodium of toad urine may reach
a level as low as 0.5 mEq/l with a mean value of 4.8 mEq/l, depending upon
the state of hydration of the animal. This reabsorptive capacity cor-
responds to that of the mammalian nephron. Thus, study of the toad urinary
bladder has assumed wider interest as a model for more complex tissues
engaged in active transport. Caution must be exercised, however, in applying
conclusions about active transport drawn from the toad bladder to other
tissues. Certain differences have been discovered between the transport
systems of the toad bladder and mammalian kidney; one of these is the
finding that the transport mechanism in the toad bladder does not appear
to carry potassium. It would seem then that the toad bladder should be
regarded as a convenient model for the development of new hypotheses to
be tested upon more complex tissues.

B. The effect of aldosterone on the urinary bladder.

The adrenal glands of Bufo marinus are present as scattered columns of acidophilic cells embedded within the ventral surface of the kidney (Vincent, 1898). The importance of aldosterone in phylogenetic development has been effectively reviewed by Denton ('65). While aldosterone had been purified from adrenal tissue as early as 1953 and quantified in human plasma by 1955 (Simpson and Tait, '55), Crabbé ('61a) was the first to demonstrate aldosterone in Bufo marinus. In quantitative terms, 0.68 µg aldosterone per 100 ml plasma were found in toads maintained in saline and 1.17 µg aldosterone per 100 ml plasma in those maintained in distilled water, values ten times greater than those of normal man (Peterson, '59). At the same time, Crabbé ('61a) reported that the isolated urinary bladder from toads receiving parenteral injections of aldosterone demonstrated increased sodium transport over toads not receiving aldosterone, the degree of increase being greater in toads maintained in distilled water. Subsequently, Crabbé ('61b) demonstrated that the addition of aldosterone to the media bathing the serosal surface of the membrane could stimulate transport of sodium in vitro. A delay of 60 minutes was found prior to the increase in transport, which reached a maximum within 120 minutes. The increase was much greater in toads maintained in distilled water prior to isolation of the urinary bladder.

Following the original use of the urinary bladder of the toad by Crabbé to study the physiological effects of aldosterone, this model system has contributed much to the understanding of the cellular basis for the mechanism of action of this important hormone. Use of the toad bladder has been instrumental in studies of the effective concentration and kinetics of the hormone, the role of intermediary metabolism in its function, its binding to cell components, and its relationship to cellular control mechanisms (see Sharp and Leaf, '66). The studies of Frazier ('62) demonstrated that the mucosal aspect of the membrane is responsible for sodium transport. Crabbé ('61b) established that the maximum stimulation in vitro of sodium transport by aldosterone is reached at a concentration of 10^{-7} M, beyond which very little additional stimulation was detected. The minimum effective concentration in vitro was found to be 3.3 X 10^{-10} M by Sharp and Leaf ('66). The hormone is nearly completely bound to the toad bladder within 30 minutes of its addition to the bathing medium, and by autoradiography using tritiated aldosterone, the hormone has been localized to the nuclei of all epithelial cells (Edelman et al., '63). Ausiello and Sharp ('68) demonstrated that the nuclear binding, presumed to represent a "receptor site", exists in at least two forms with differing dissociation constants. It is not known whether these different binding sites are present in the same nucleus or whether they represent binding by different cells.

Because aldosterone induces increased uptake of tritiated uridine simultaneously with the increased transport of sodium, and because Actinomycin D blocks the stimulation of active transport by aldosterone, Edelman et al. ('63) concluded that aldosterone acts by stimulating DNA dependent RNA synthesis to produce a messenger for the formation of a new protein. Edelman and associates ('64) further theorized that this protein might be an enzyme intimately connected with the oxidative phosphorylation of ADP to provide a high energy intermediate to drive the "sodium pump". Part of the evidence adduced for the latter theory was an increased content of ATP found by biochemical assay under conditions of increased active transport (Edelman, '64) and the finding that the substrate depleted bladder fails to respond to aldosterone with increased active transport unless supplied with a basic substrate such as pyruvate (Edelman et al., '63). Fanestil and Edelman ('66) also concluded that the mechanism for the basal rate of sodium transport differs from that of aldosterone stimulated transport since the former is not inhibited by Actinomycin D.

A contrasting theory has been proposed by Sharp and Leaf ('66) who found that under the influence of aldosterone the toad bladder displayed increased uptake of the substrates pyruvate and acetoacetate when sodium was present in the bathing medium over control tissue in which sodium was absent. This finding led them to conclude that energy metabolism was secondary to the active transport process in the toad bladder. Additional support for this concept arose from the discovery that Amphotericin B

which also stimulates sodium transport required the presence of substrate even though the antibiotic was not implicated in protein synthesis. This suggested that utilization of the mechanism of energy production is a non-specific aspect of the active transport process. They and Bonting and Canady ('64). moreover, were unable to confirm the finding of increased ATP levels under conditions of aldosterone stimulated active transport. Sharp and Leaf ('66) favor the view that a product of aldosterone induced protein synthesis, possibly an enzyme, increases the permeability of the mucosal cell membrane to sodium in a morphologically polarized manner such that sodium ions are drawn off from the serosal aspect of the mucosal cell into the extravascular space. Cuthbert ('68) however who developed an electricophysiological method to seperate permeability from active transport was not able to demonstrate increased permeability of the toad bladder after blockage of the sodium pump with ouabain and treatment with aldosterone. To date, no such permease has been isolated, and the evidence for its presence is indirect at the present time. Neither of the two major theories of aldosterone action, however, is incompatible with the other (Fanestil, '69).

C. Morphology of the toad urinary bladder.

Upon entering the abdominal cavity of the toad from the ventral aspect, the urinary bladder is easily found as a bilobed transparent

vesicle arising from the cloaca. Varying with the state of hydration,
the fluid-filled bladder may occupy a large proportion of the abdomen,
at times reaching the costal margin. Histologically, three major zones
can be identified from the peritoneum inward to the luminal or interior
surface of the bladder: first, a uniform layer of flattened squamous
cells, the serosa; second, the lamina propria, composed of interwoven
bands of collagen fibers containing scattered fasicles of smooth muscle,
a rich vascularization and myelinated nerves; and third, the mucosal
epithelium. It has been known since the late nineteenth century from
standard histological observations (Schiefferdecker, 1884) and more
recently from electron microscopic studies (Peachey and Rasmussin, '61;
Choi, '63) that the epithelium is not a homogenous population of cells.
Indeed, the mucosa is made up of several cell types: granular cells,
mitochondria-rich cells, goblet cells, and basal cells. The most abund-
ant are the granular cells, so-called because of their diastase resistant,
periodic acid-Schiff (PAS) positive, subapical granules. About ten per
cent of the cells are the mitochondria-rich cells, which can be identified
with the phosphotungstic acid-hematoxylin (PTAH) stain for mitochondria
and with histochemical methods for the dehydrogenase systems. A small
percentage of the cells of the mucosa are goblet cells filled with PAS
and Alcian blue positive material. A small basal cell with a bilobed
nucleus is attached to the basement membrane but is not exposed to the
lumen.

At the electron microscopic level (see Choi, '63), the luminal
surface of all cells contains numerous microvilli. The characteristic
granules of the granular cells are encased in a membrane, occasionally
confluent with the plasma membrane and the endoplasmic reticulum; the
granules also contain a fine fibrillar meshwork. The mitochondria-rich
cell is distinguished electron microscopically by its abundant large
mitochondria and its exposure to the luminal surface in the form of a
peninsula of mitochondria filled cytoplasm. Both granular and mitochondria-
rich cells possess numerous vesicles near the basement membrane. Both
are also connected by tight junctions. The goblet cell is composed of
abundant droplets containing electron-dense material. The basal cell
is marked by the sparseness of its cellular components and its increased
presence of filaments. DiBona et al. ('69) have demonstrated with serial
electron microscopic sections that the granular and mitochondria-rich
cells may lie directly upon the basement membrane of the toad bladder.
This confirms the light microscopic and electrophysiological evidence
that the urinary bladder may be considered as composed of a single cell
layer over much of its surface.

D. Scope of the present investigation.

As reviewed above, the toad urinary bladder is felt to represent
in many ways an extension of the nephron, functioning as a homeostatic

unit controlled by aldosterone. Evidence has accumulated that the epithelium of the bladder is the active component in the sodium transport mediated by aldosterone and is the target site of the hormone. While localization of aldosterone function at the subcellular level has made much progress, the methods utilized have not provided direct evidence concerning the role of the several epithelial cell types in active transport. Thus, the specific cell types in the epithelium affected by aldosterone are not definitely known. Since the epithelial cell types can be distinguished by histochemical means, which delineate morphology and to a more limited extent function, changes in staining patterns following administration of aldosterone might indicate cellular sites of action of the hormone. Thus, the present studies describe the histochemical changes observed in the mucosa following stimulation by aldosterone, under in vitro and in vivo conditions. In addition, biochemical studies on the influence of aldosterone upon oxidative phosphorylation of toad bladder mitochondria were performed and correlated with the histochemical investigations.

MATERIALS AND METHODS

A. Histochemistry

Twenty-four medium sized female toads, Bufo marinus, kept in moist terraria were employed in the following study. The protocol consisted of two parts, in vitro experiments in which aldosterone was incubated with the excised bladder, and in vivo experiments in which aldosterone in four doses was given parenterally prior to the removal of the bladder.

For the in vitro experiments, the toads were kept in 0.6% saline for 24 hours before pithing and excision of the bladder (Porter and Edelman, '64). During the entire course of the in vivo experiments, the toads were kept in 0.6% saline. The bladders were removed, bisected, immediately immersed and washed in cold Ringer's solution (made with 0.6% NaCl). In the in vitro studies, one hemibladder was incubated in a control solution of Ringer's with 2 mM pyruvate (Sharp and Leaf, '64); the other incubated in Ringer's with 2 mM pyruvate and 1×10^{-7} M aldosterone (obtained from Sigma Chemical Co. and assayed for electrical activity upon the toad bladder; see Appendix). Five mg of the hormone were dissolved in ethanol and diluted with 0.6% saline to 5 ml, from which appropriate dilutions were made. Incubations were carried out for three hours in a Dubnoff metabolic shaker at 20°C.

In the _in vivo_ experiments, 1 ml of 5 X 10^{-6} M aldosterone was injected intraperitoneally every 12 hours for 48 hours, at the end of which time the animals were sacrificed. As a control, 1 ml of the excipient was injected into other toads upon the same schedule, since hemibladders could not be used for control tissue in this case.

Enzymatic histochemical reactions were carried out upon fresh tissue, while paraffin sections were made from tissue fixed in Lillie's neutral buffered formalin (Lillie, '54). Both cryostat sections (frozen in cyclohexane and cut at -20°C) and whole mounts stretched over wax impregnated corks with elastic bands were made with fresh tissue. Following incubation in the histochemical reaction media, the tissue was fixed in Lillie's neutral buffered formalin for 20 minutes, washed for ten minutes in running water and mounted in glycerine. The use of whole mounts permitted more thorough interpretation of cellular relationships and distribution.

Paraffin sections were stained with Harris hemalum and eosin (H&E), the PAS reaction with and without diastase treatment (McManus and Mowry, '60), the PTAH stain (Mallory, 1897), and the methyl green-pyronin reaction with and without ribonuclease treatment with 30 minute incubation (Pearse, '60).

The localization of adenosine 5-triphosphatase (ATPase) activity was studied on fresh and formalin fixed tissue with the lead method of

Wachstein and Meisel ('57). Fixed tissue was incubated for 60 minutes; fresh tissue, for 20 minutes. The method of Wachstein and Meisel ('56) was used for glucose 6-phosphatase activity with 20 minute incubation at $37^{o}C$.

Histochemical reactions for triphosphopyridine and diphosphopyridine nucleotide diaphorase (TPND and DPND) activity were carried out by the methods of Nachlas et al. ('58). The incubation was carried out at $37^{o}C$; 15 minutes for TPND and 30 minutes for DPND with Nitro BT (obtained from Sigma Chemical Co.). Succinic dehydrogenase (SDH) activity was localized after the method of Nachlas et al. ('57) with 30 minute incubation at $37^{o}C$. Lactic dehydrogenase (LDH), glucose 6-phosphate dehydrogenase (G6PD), and 6-phospho-glucuronate dehydrogenase (6PGD), activities were demonstrated after the methods of Hess et al. ('58) with incubation times of 20 to 30 minutes.

The G-Nadi reaction for cytochrome oxidase activity was performed according to the method of Burtner and Lillie ('53) with 20 minutes incubation. Non-specific esterase activity was studied with $\propto$-naphthol acetate and fast blue B salt after Gomori ('50).

Specificity of enzymic reactions was examined by omission of appropriate substrate from the incubation media. In all cases where quantitative observations were made, control tissues were incubated along with the experimental tissues in the same media concurrently.

B. Biochemistry -- Oxidative phosphorylation.

The oxidative phosphorylation of mitochondria under three sets of
conditions was studied: mitochondria from control bladders, mitochondria
treated with aldosterone without incubation after isolation from control
bladders, and mitochondria from bladders incubated with aldosterone three
hours prior to isolation.

Prior to pithing and excision of the bladders, toads were maintained
in 0.6% saline. In the first series of experiments, mitochondria were
harvested directly from the urinary bladder after washing several times
in ice chilled saline. In the second series, after washing several times in
saline the bladders were incubated with 10^{-7} M aldosterone and 2 mM pyruvate
in 50 ml of saline for three hours at 20°C. Mitochondria were harvested
by scraping the mucosa with a clean glass slide; the scrapings from
six bladders were pooled for each experiment. Scrapings were homogenized
in .25 M sucrose with 2 mM EDTA in a Dounce type homogenizer with three
strokes of the piston. The homogenate was layered over .35 M sucrose
and centrifuged at 1000 X g for ten minutes to sediment nuclei and debris.
The supernate was then centrifuged at 8000 X g for ten minutes to con-
centrate the mitochondria which were washed three times by resuspending
the pellet in .35 M sucrose and centrifuging at 8000 X g for ten minutes.
The mitochondrial pellet was then resuspended in 1.42 M sucrose and
layered over a sucrose density gradient of 1.42 M to 1.88 M sucrose which
was stored at 0° C for 12 hours prior to the addition of the mitochondria.

This was centrifuged at 39,000 X g for two hours in a type 5W50L
Spinco swinging bucket rotor. Allowing the gradient to leave the tube
through a small perforation in the bottom, the mitochondrial fraction
(located approximately 2/5 of the length of the tube measured from the
top) was captured and resuspended by pipetting.

Oxygen uptake of the mitochondria was measured in a polarograph
with an oxygen electrode (Clarke type) at $30^{\circ}C$, recording at a speed of
1 inch/ minute, each inch of deflection for O_2 uptake corresponding to
10 µatoms of oxygen. Constant agitation in the 1.5 ml cell was maintained
with a magnetic micro stirring bar.

The media contained:

        125 µmoles   sucrose
         25 µmoles   Tris phosphate buffer   (pH 7.4)
        .75 µmoles $MgSO_4$

to which was added, first the mitochondrial suspension (3 mg protein,
.1 ml); secondly, 50 µmoles glutamate-malate substrate as the potassium
salt; thirdly, 460 µmoles of ADP, the Barium salt adjusted to pH 7.4
with Tris buffer. Each of the three latter additions took place after
the media was allowed to equilibrate in the chamber.

RESULTS

A. Histochemistry

H & E

This stain revealed no obvious difference in the histology of control and in vitro treated tissue. In the in vivo treated tissue, approximately ten per cent of the cells were binucleated. This was especially apparent in the whole mount preparations.

PAS

Two types of staining within the epithelium are prominent with this reaction in control tissue: the opaque particles of the granular cells that are diastase resistant, and a faint diffuse tincture of the cytoplasm abolished by diastase treatment (fig. 1). Moderate increase of the latter type of staining of the cytoplasm, especially near the luminal border of the bladder, were seen with in vitro treatment with aldosterone (fig. 2). The increase in the cytoplasmic staining was even more marked in the in vivo treated material (fig. 3). Moreover, the PAS positive diastase resistant granules were smaller and less numerous among the granular cells. Goblet cells were intensely stained (figs. 1-3).

PTAH

In control tissue, the cytoplasm of the mitochondria-rich cells
showed diffuse basophilic granulations characteristic of mitochondria
(fig. 4). The same pattern was observed after in vitro treatment with
aldosterone (fig. 5). Under in vivo conditions, more basophilic granules
were seen in the cytoplasm of the granular cells, especially in the
perinuclear region (fig. 6).

METHYL GREEN-PYRONIN

The cytoplasm of all cells in control tissue stained a uniform
faint reddish hue (fig. 7), which was abolished by ribonuclease (RNAase)
treatment of the sections. In tissues treated with aldosterone in vitro
and in vivo, the staining was a distinctly deeper shade of red, pyrino-
philia reaching a peak intensity at three hours (fig. 8), and remaining
at the same degree under more prolonged treatment of in vivo conditions
(fig. 9).

DPND and TPND

The diaphorase activities of the reactions which utilized Nitro BT
were found to be localized diffusely in the cytoplasm of different cells
as well as intensely within irregular-sized, coarse granules. Control
tissue displayed its most intense reaction within mitochondria-rich cells

which were packed with reactive granules (fig. 10). Granular cells showed some variation in staining with these reactions, but were not nearly as reactive as the mitochondria-rich cells, although they too contained reactive granules (fig. 10). Few changes were observed with in vitro treated tissue (fig. 11), but under in vivo conditions, not only were the intensely reactive mitochondria-rich cells increased in number, but the granular cells contained increased numbers of reactive granules in varying degrees (fig. 12).

SDH

In control tissue, intensely reactive mitochondria-rich cells were situated against a background of less reactive granular cells (figs. 13, 15). Reaction products were localized to coarse cytoplasmic granules (figs. 13, 15). This difference between mitochondria-rich and granular cells was likewise observed under in vitro conditions. Granular cells did not appear to contain increased numbers of reactive granules. On the other hand, the in vivo experiments showed marked differences over controls: not only were the normally intensely reactive cells more numerous, but previously weakly reactive cells showed gradations in intensity of staining, some being so reactive as to make the distinction between cell types less apparent (figs. 14, 16).

CYTOCHROME OXIDASE

Results with this reaction were similar to those for SDH; mito-
chondria-rich cells were clearly differentiated from the sparsely staining
granular cells in control tissue (fig. 17), a relationship unchanged
under in vitro conditions. The in vivo treated tissue, however, showed
more of the heavily reactive cells as well as increased reactivity of
the granular cells (fig. 18).

LDH

This enzyme was found in control tissue to show a diffuse localization,
with perhaps a slight predeliction for the mitochondria-rich cells; no
cells displayed very strong activity for this enzyme. After treatment
with aldosterone for three hours in vitro, no appreciable increase in
the number of reactive granules was seen in the mucosa. A very mild
increase in reactivity, primarily of the granular cells, was seen under
in vivo conditions, however.

G6PD and 6PGD

In control tissue, activity of these enzymes was localized diffusely
in the mucosal cytoplasm, as well as within small granules (figs. 19, 22).
Scattered clusters of cells were intensely reactive (figs. 19, 22).
After in vitro incubation with aldosterone for three hours, the sparse

clusters of reactive cells were found in the same distribution but were
more intensely reactive in comparison to cells in control tissue (figs. 20,
23). In the in vivo experiments, the clusters of intensely reactive cells
became larger and more numerous, the configurations assuming a stellate
appearance (figs. 21, 24).

ATPase

The lead nitrate reaction for ATPase was carried out with both formol
fixed and fresh tissue. Consistent with previous reports (Keller, '63),
activity was localized to the region of the mucosa adjacent to the base-
ment membrane in fixed tissue; in fresh tissue, activity was localized
both along the entire membrane and within the cytoplasm (fig. 25). Cyto-
plasmic activity was particularly apparent in the mitochondria-rich cell.
This pattern was found both in control and in in vivo treated tissue
and repeated trials showed no increased activity over control tissue.
In vivo treated tissue, however, showed more intense ATPase activity along
the entire cell membrane as well as more cytoplasmic activity (fig. 26).

GLUCOSE-6-PHOSPHATASE

Control tissue showed localization of this enzyme activity within
perinuclear particles and to the cell membrane of both mitochondria-rich

cells and the granular cells (fig. 27). After in vitro treatment, the

perinuclear activity was moderately increased (fig. 28). Following

aldosterone treatment in vivo, activity for this enzyme was found in

the same location with a slightly greater intensity of reaction.

NON-SPECIFIC ESTERASE

Intense esterase activity in control tissue was found to be limited

to a small population of cells, possibly the mitochondria-rich cells;

in other cells it was seen within vacuoles in the periphery of the cyto-

plasm adjacent to the cell membrane (fig. 29). In in vitro and in vivo

treated tissue, not only were these intensely staining cells even more

reactive, but the peripheral cytoplasm of the remaining cells was also

more reactive (fig. 30).

B. Biochemistry -- Oxidative phosphorylation.

Illustrations of several of the recordings from the polarograph are provided in figs. 31-33 in which the effect of the addition of various substrates upon the respiring mitochondria is clearly seen.

The results of the first series of experiments is summarized in Table I; here the mitochondria freshly isolated were studied when aldosterone was added without incubation. A small decrease of the P/O ratios was found in this case. It is also significant that the rate of oxygen consumption (that portion of the curve labelled State IV; compare figs. 31, 32) was greater than control mitochondria.

TABLE I

	P µmoles ADP	O µatoms	P/O	% decrease	State III State IV
Control	460	165	2.89	9.6	5/.9 = 6
Aldo	460	175	2.61		6/5 = 1.1
Control	460	150	3.06	16.1	7/2 = 3.5
Aldo	460	180	2.55		9/7 = 1.3
Control	460	155	2.98	17.2	10/.9 = 11
Aldo	460	185	2.42		8/5 = 1.6

The results of the second series of experiments is summarized in Table II; here mitochondria were isolated from bladders which had been incubated with aldosterone for three hours and from control bladders. Moderate decreases in P/O ratios were observed for aldosterone treated tissue over controls, the per cent decreases ranging from 31 to 49.

TABLE II

	P μmoles ADP	O μatoms	P/O	% decrease	State III / State IV
Control	460	190	2.42	31	9/1.5 = 6
Aldo	460	280	1.63		4/.8 = 5
Control	460	170	2.70	37	9/1.1 = 10
Aldo	460	270	1.71		8/2.1 = 4
Control	460	145	3.20	49	4/.3 = 12
Aldo	460	285	1.62		5/2 = 2.5

DISCUSSION

The mitochondria-rich cells were found normally to have more activity
for the citric acid cycle and respiratory enzymes, while granular and
goblet cells were much less reactive. These results are in agreement
with Keller's ('63) observations on the basic histochemistry of the toad
bladder. The findings here reported show in addition that increases in
the enzymes of certain metabolically significant pathways occur after
exposure of the toad bladder to aldosterone. Furthermore, after pro-
longed in vivo exposure, alteration in histochemical cellular relationships
could be detected.

Treatment of the toad bladder in vitro with aldosterone using the
concentration and timespan employed by physiologists to demonstrate
maximal sodium transport produces a marked pyrinophilia in all cells which
is abolished with RNAase treatment. The intensity of the pyrinophilia
reaches its maximum concurrently with the maximum stimulation of sodium
transport, namely at 120 minutes. Pyrinophilia similarly increased
following in vivo administration of the hormone. This finding is con-
sistent with the increased RNA production known to occur with the in-
duction of sodium transport (Edelman et al., '64). The histochemical
studies show that the increase in RNA production following aldosterone
stimulation occurs in all cell types. Thus, the evidence indicates

that aldosterone is present intracellularly in all cell types after
exposure to aldosterone (see Edelman et al., '64) and that all cell
types respond histochemically concurrently with the earliest biochem-
ically detectable phase of altered metabolic function.

The enzymes of the hexosemonophosphate shunt were also found to
be increased in the in vitro situation, and even more so in vivo. On
the basis of the arrangement of the cells in stellate clusters, as
well as the shape of the cells, these reactive cells were felt to include
more than the mitochondria-rich cells. The increased activity in the
hexosemonophosphate shunt, also demonstrated biochemically by Handler
et al. ('69), may represent a response to increased metabolic demands upon
the cells, or more likely in view of the increased RNA production noted
above, may be related to increased synthesis of the precursors of nucleic
acids (Pearse, '60). The finding of activity of these normally cyto-
plasmic enzymes both in the cytoplasm and in the form of granules approx-
imately the size of the granular cell PAS positive diastase resistant
particles raises the possibility that the enzymes may be present within
these particles. The significance of the pattern of increases in the
"shunt" enzymes, namely in closely adjacent cells, is unclear at present.

Leaf et al. ('59) have shown that oxidative metabolism is essential
to aldosterone-stimulated transport of sodium, although baseline transport
of sodium will continue under anaerobic conditions. Likewise, oxygen

consumption is increased with aldosterone induced sodium transport
(Sharp and Leaf, '66). Numerous studies (Edelman et al., '63; Sharp
and Leaf, '65) have demonstrated the substrate dependent nature of
aldosterone action; once endogenous substrates have been depleted
increased use of metabolites such as pyruvate occurs. Recently, Kirsten
et al. ('68) have demonstrated significant increases in the mitochondrial
tricarboxylic acid cycle enzymes after incubation with aldosterone
for 15 hours. These enzymes included condensing enzyme, isocitrate
dehydrogenase, glutamate dehydrogenase, and malate dehyrogenase.

Histochemically, marked increases in oxidative enzyme activity were
observed after in vivo treatment with aldosterone, but not in vitro.
The failure to demonstrate increased activity of the enzymes in the
in vitro experiments may reflect one or more of the following: inability
of the severed bladder to respond with augmented oxidative machinery,
the removal of these steps of intermediary metabolism from the initial
target of aldosterone action, or the failure of the histochemical methods
for these enzymes to demonstrate small increases in activity, particularly
in the normally intensely reactive mitochondria-rich cells. The latter
possibility is made more likely by the small but significant rises in
assays of the tricarboxylic acid cycle enzymes found after three hours
in vitro incubation with aldosterone by Kirsten et al. ('68). Under more
prolonged aldosterone stimulation, however, the diffuse histochemical

increases in the oxidative machinery reflected by SDH, DPND, TPND, and
cytochrome oxidase activities is in complete agreement with the recent
biochemical studies on similar enzymes. Another reflection of heightened
metabolic activity is the increase in diastase labile PAS staining,
presumably glycogen.

The quantitative histochemical increases then support the physiological
and biochemical evidence that aldosterone stimulates oxidative metabolism.
The relatively small changes seen in LDH activity after aldosterone stim-
ulation further support the concept that this hormone primarily affects
aerobic processes. This histochemical finding is also consistent with the
results of Kirsten et al. ('68) who demonstrated no significant increase
in LDH under in vitro conditions. The demonstrated increase in the enzymes
of the tricarboxylic acid cycle may be directly related to aldosterone
function. These enzymes are stimulated by aldosterone even when sodium
transport is prevented by removal of sodium from the mucosal medium. Thus,
aldosterone seems to independently increase the activity of these enzymes
and to stimulate sodium transport (Kirsten et al., '68).

The relationship of ATPase to the mechanism of aldosterone stimulated
active transport has been a focus of interest since the discovery by Skou
('62) of a sodium activated ATPase system and its linkage to active
transport. Although the specificity of the ATPase reaction has been
questioned (Barka and Anderson, '63), with the method employed ATPase

activity was found to be localized to the cell membrane (see also
Bartoszewicz and Barrnett, '64), and to granules in the mitochondria-
rich cells of control tissue. Under in vitro conditions, no histo-
chemical alterations were observed over control tissue, a finding
consistent with the biochemical assays of Sharp and Leaf ('66) and
Bonting and Canady ('64). These investigators found no increases in
ATPase levels under similar conditions, nor increases in ATP after
blockage of the sodium pump with ouabain, results which indicated no
direct association of ATPase with the active transport system. Pro-
longed stimulation with aldosterone, however, did result in notable
histochemical increases, primarily in the cytoplasm adjacent to the
cell membrane within the granular cells. Indeed, the granular cells
under these conditions appeared to be nearly as reactive as mitochondria-
rich cells in control tissue. Whatever the precise role of ATPase in
the active transport system and its response to aldosterone may be,
the nearly unvaried occurrence of this enzyme within epithelia engaged
in active transport (Heinz, '67) provides a rather constant morphological
characteristic of such tissue.

Several possible pathways by which aldosterone might control energy
metabolism exist. Evidence has already been presented that it mediates
the production of key enzymes in the tricarboxylic acid cycle. A second
possibility has been shown to be less likely, namely an increase in ATPase

activity. A third possibility has received little attention: that

aldosterone may affect the respiratory transport chain. Histochemically,

the increases seen in cytochrome oxidase, DPND and TPND after aldosterone

stimulation support this concept. In a series of experiments to test

this hypothesis further the utilization of oxygen under ADP (adenosine

diphosphate) controlled respiratory states was examined in mitochondria

isolated from the toad urinary bladder. This method, introduced by

Chance and Williams ('56) consists of adding a known amount of ADP and

an excess of substrate to a suspension of mitochondria while measuring

the uptake of oxygen. An assumption is made that a high energy inter-

mediate is formed which is converted to ATP. By computing the ratio of

moles of ADP to the moles of oxygen utilized (the "P/O" ratio) a value

for each substrate may be arrived at: comparison with the theoretical

value for this substrate allows a conclusion concerning the respiratory

state of the mitochondria to be made.

The results of these experiments indicate that aldosterone does

affect the electron transport chain. A mild decrease in the P/O ratio

was noted when mitochondria were exposed directly to the hormone, possibly

a non-specific effect of the steroid nucleus. Furthermore, a more marked

decrease was consistently observed in mitochondria isolated from bladders

which had been incubated with aldosterone. The latter mitochondria

would be exposed to the hormone mediated metabolic control agents, unlike

those in the first series of experiments, but they would also be in

contact with the hormone itself for a longer period of time. This

increased utilization of oxygen per mole of ADP indicates a less tightly

coupled respiratory state of the transport chain. It should be noted that

the theoretical P/O ratio for the substrate is 4.0 and since the highest

value in control mitochondria was 3.2, it would seem that native toad

bladder mitochondria exist in a somewhat loosely coupled state also.

One possible explanation of the aldosterone uncoupling mechanism is an

increased catalysis of the ATP-ADP exchange reaction discovered by Wadkins

and Lehninger ('63) and thought to be the terminal event in oxidative

phosphorylation. Theoretically at least, increased turn-over of a high

energy intermediate could contribute to the active transport process by

supplying an easily accessible, if perhaps transient, energy source.

Reversibility of this system would account for the failure to find increased

ATP levels. Heightened activity of the electron transport chain may there-

fore represent yet another effect of aldosterone upon oxidative metabolism.

The histochemical approach is particularly valuable in elucidating

the cellular distribution of the increase in the oxidative machinery.

The results of the in vivo experiments indicate that the burden of increased

sodium transport in the toad bladder is levied upon a wider population of

cells than the mitochondria-rich cells, namely the granular cells.

Following aldosterone administration, mitochondria-linked enzymes were

no longer neatly compartmentalized into the mitochondria-rich cells, but increased activity was found in a graded fashion in the granular cells, identified as such by their diminished but still present PAS positive granules. The indication of an increased number of mitochondria in these cells was supported by PTAH staining.

The greater number of reactive mitochondria could be related to an increase in the number or the activation of enzymes in pre-existing mitochondria. Kirsten et al. ('68) also found an increase in enzyme activity per mg of protein of the mitochondrial fraction of the cell homogenate. This finding is consistent with activation of pre-existing enzymes or an increase in concentration of enzymes within mitochondria and does not rule out simultaneous increases in the number of mitochondria.

Thus, the granular cell approaches the histochemical profile of the mitochondria-rich cell under prolonged aldosterone stimulation. If we can infer that it acquires the physiological potential as well, the granular cell may assume added responsibility for the increased sodium transport which follows aldosterone stimulation. The above conclusion is consistent with that of certain radio-kinetic studies (Hoshiko and Ussing, '60; Frazier et al., '62) indicating that the number of mitochondria-rich cells is too small to account for increased inclusion of radio-active sodium (see also Keller, '63). The histochemical studies, therefore, indicate that aldosterone has a striking influence on the morphological as well as the biochemical phenotype of the cell.

SUMMARY

Histochemical changes were observed in the epithelium of the
toad urinary bladder following stimulation by aldosterone under in
vitro and in vivo conditions to determine the cell types affected
by this hormone. Under in vitro conditions, a marked pyrinophilia
in comparison to control tissue affecting all cell types was observed,
as well as slight increases in cytoplasmic PAS staining within the
granular cells. Granular cells were likewise slightly more reactive for
G6PD and 6PGD than control tissue, whereas enzymes of the oxidative
pathways and ATPase were unchanged under in vitro conditions. In
addition, oxidative phosphorylation in mitochondria isolated from
bladders stimulated with aldosterone was noted to show a looser state
of coupling than in that of control tissue. Under in vivo conditions,
pyrinophilia persisted over control tissue, and PAS cytoplasmic
staining was increased. Activity of ATPase was more intensely local-
ized to the cell membrane and cytoplasm of all cells, while more cells
were heavily reactive for hexosemonophosphate shunt enzymes than under
in vitro and control conditions. The most significant changes under
in vivo conditions appear to be the marked increases in the enzymes of
the oxidative pathways and the increased number of mitochondria demon-
strated with the PTAH stain within the granular cells. Thus, under

prolonged aldosterone stimulation, the granular cell approached the

histochemical profile of the mitochondria-rich cell. The hormone

may therefore act upon the granular cell, which comprises the largest

number of cells within the epithelium, as well as upon the mitochondria-

rich cell.

APPENDIX[1]

The physiological activity of the preparaton of aldosterone utilized
was confirmed by the short circuit technique. A hemibladder from the same
toad was mounted in each side of a Lucite dual chamber containing electrodes
and toad Ringer's solution without glucose; the bladder was allowed to
equilibrate within the chamber 17 hours prior to the assay. The urinary
bladder employed was excised from a toad which had lived in saline for
four days. Four hours before assay, fresh toad Ringer's with .1 M glucose
was exchanged, and at time "0", an amount of aldosterone sufficient to
bring the concentration to 10^{-7} M was added to the serosal half of the
chamber. At ten minute intervals the short circuit current was recorded
for the control and the aldosterone treated bladder. The results are
reported in graphic form in fig. 34, where:

$$\frac{SCC_t}{SCC_O} = \frac{\text{short circuit current at time x}}{\text{short circuit current at time o}} .$$

The aldosterone treated bladder showed the expected rise in current with
the proper time relationship for a potent sample of the hormone. Thereafter,
the same preparation, stored at 4° C, was used in all studies.

1. The above work was done with the kind collaboration of Dr. Arthur Finn,
Department of Physiology, Yale University School of Medicine.

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EXPLANATION OF FIGURES
Plate   I

Figure 1. PAS stain, control, paraffin section. Goblet cells (G) are intensely stained and granular cells (Gr) contain PAS positive particles as well as faint cytoplasmic staining. (X 1175)

Figure 2. PAS stain, in vitro aldosterone, paraffin section. Cytoplasmic staining is moderately increased over control tissue, especially near the luminal border (top). (X 1500)

Figure 3. PAS stain, in vivo aldosterone, paraffin section. Cytoplasmic staining is more intense than in both control and in vitro treated tissue. Note the binucleate cell (arrow). (X 1875)

Figure 4. PTAH stain, control, paraffin section. The cytoplasm of most cells is faintly stained. At least one mitochondria-rich cell (M) can be identified by the more dense particularity of its cytoplasm. The cell with the clear cytoplasm is probably a goblet cell (G). (X 1875)

Figure 5. PTAH stain, in vitro aldosterone, paraffin section. No difference in staining is apparent between this and the preceding control section. (X 1875)

Figure 6. PTAH stain, in vivo aldosterone, paraffin section. The cytoplasm of all cells shows an increase in particulate staining. G, goblet cell. (X 1875)
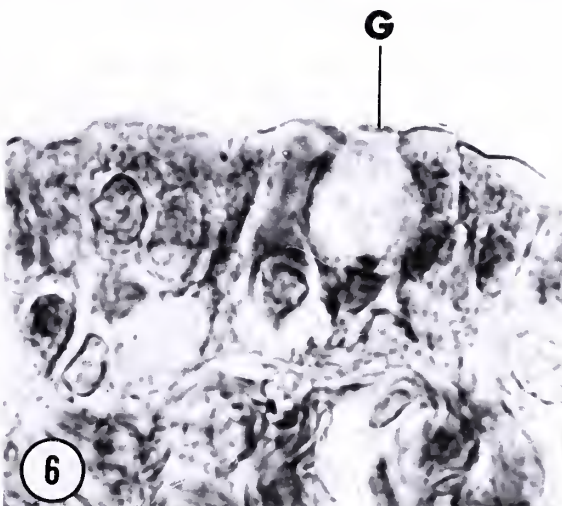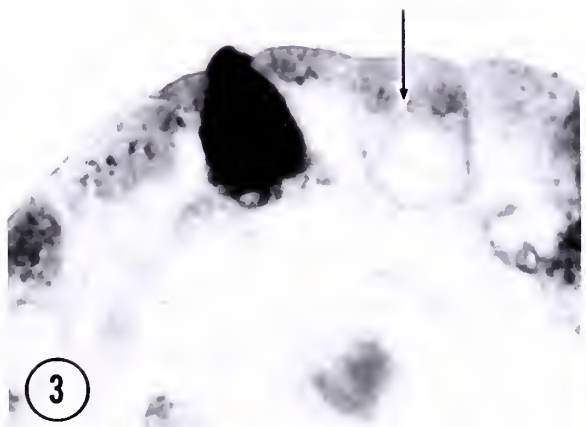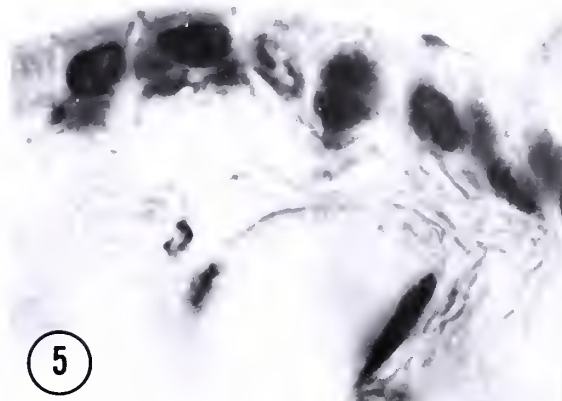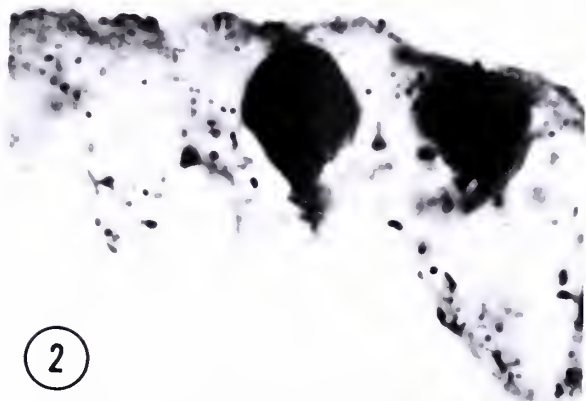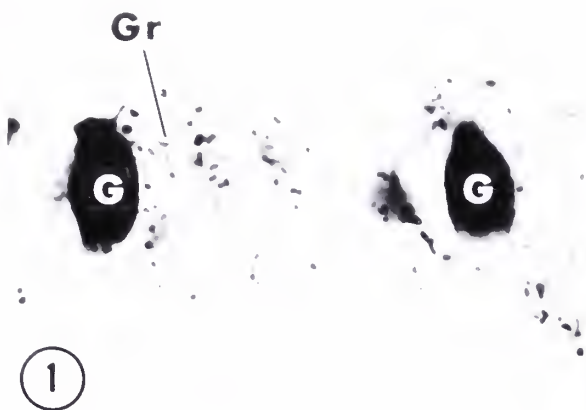
EXPLANATION OF FIGURES

Plate   II

Figure 7. Methyl green-pyronin, control, paraffin section.   Uniform
faint cytoplasmic staining is seen in all epithelial cells.   (X 1175)

Figure 8. Methyl green-pyronin, in vitro aldosterone, paraffin section.
Cytoplasmic staining is more intense in epithelial cells in comparison
to control tissue.   (X 1175)

Figure 9. Methyl green-pyronin, in vivo aldosterone, paraffin section.
Epithelium is more intensely stained than control tissue, but similar
to in vitro treated tissue.   (X 1175)

Figure 10. DPND, control, whole mount.   Intensely reactive cells
(mitochondria-rich cells) are sparsely scattered among more weakly
and variably reactive cells.   Activity is localized to small cyto-
plasmic granules.   (X 1175)

Figure 11. DPND, in vitro aldosterone, whole mount.   Few differences
are observed between this and control tissue.   (X 1150)

Figure 12. DPND, in vivo aldosterone, whole mount.   A greater number
of intensely reactive cells can be noted over the preceding figure,
as well as increased number of stained granules within the granular
cells.   (X 1150)
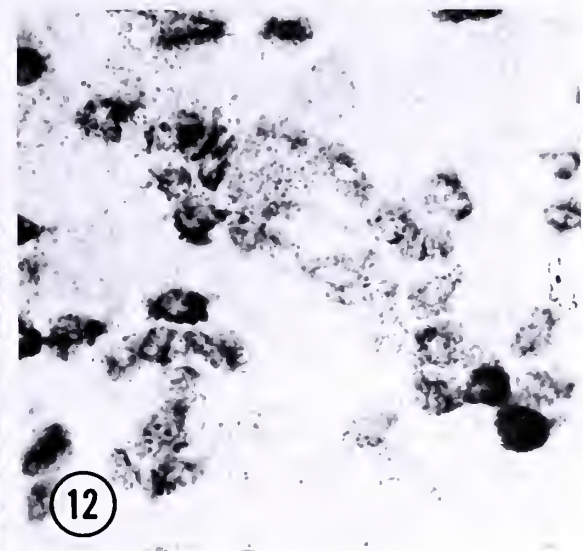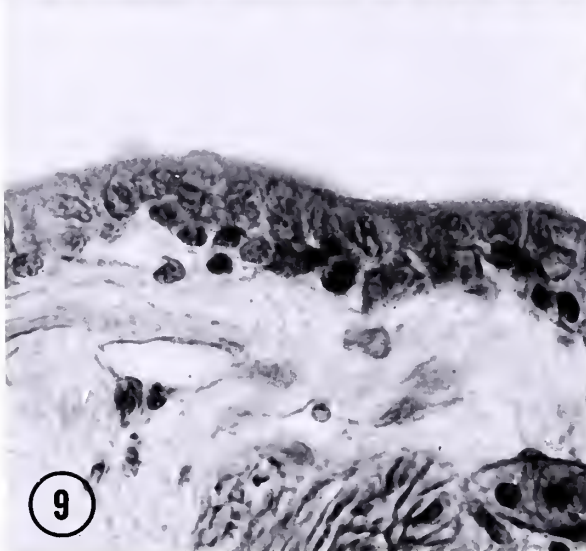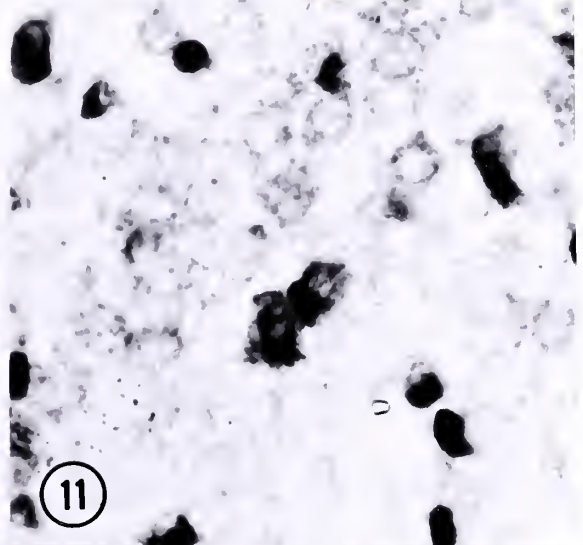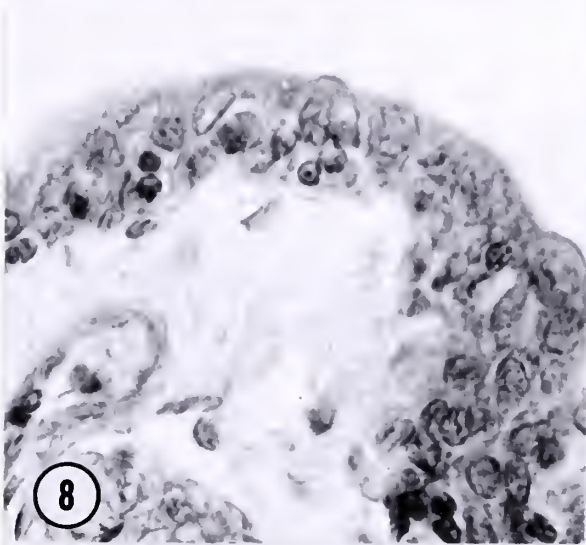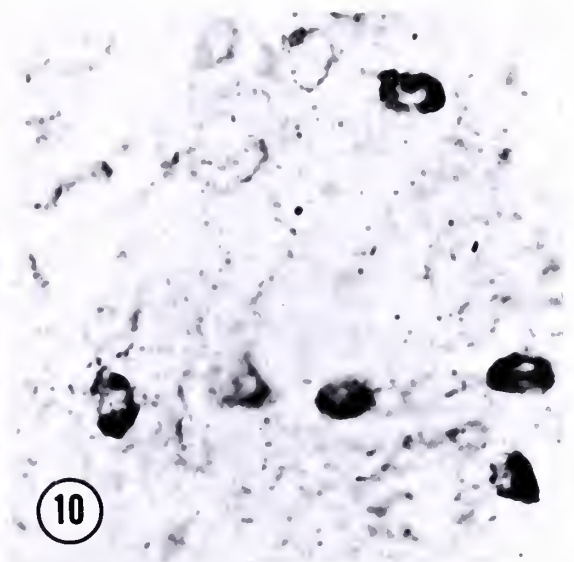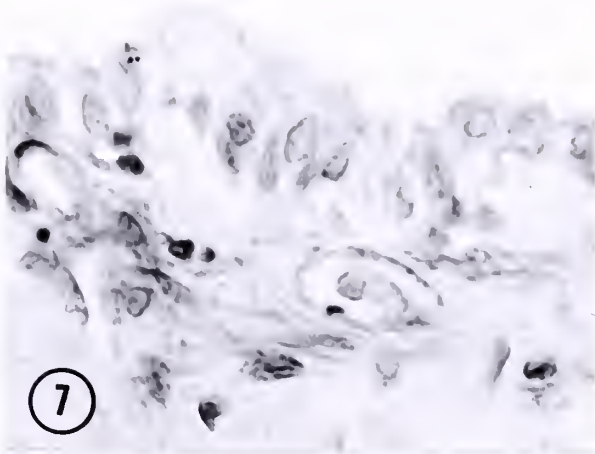
EXPLANATION OF FIGURES

Plate III

Figure 13.   SDH, control, whole mount.   Mitochondria-rich cells are filled with intensely stained granules, while granular cells contain a much smaller number of stained granules.   (X 2500)

Figure 14.   SDH, _in vivo_ aldosterone, whole mount.   Granular cells contain a larger number of stained granules than in control tissue. M, mitochondria-rich cell.   (X 2500)

Figure 15.   SDH, control, frozen section.   Note the intensely reactive mitochondria-rich cells and their distribution in the epithelium. (X 2000)

Figure 16.   SDH, _in vivo_ aldosterone, frozen section.   Increased numbers of intensely stained mitochondria-rich cells can be seen in comparison to control tissue.   (X 1875)

Figure 17.   Cytochrome oxidase, control, whole mount.   Mitochondria-rich cells (M) contain large numbers of stained granules while granular cells are weakly reactive.   (X 2100)

Figure 18.   Cytochrome oxidase, _in vivo_ aldosterone, whole mount. Granular cells are more reactive and the number of stained granules approaches the number seen in mitochondria-rich cells (M).   (X 2350)

(13)

(16)

(14) M

(17) M

(15)

(18) M
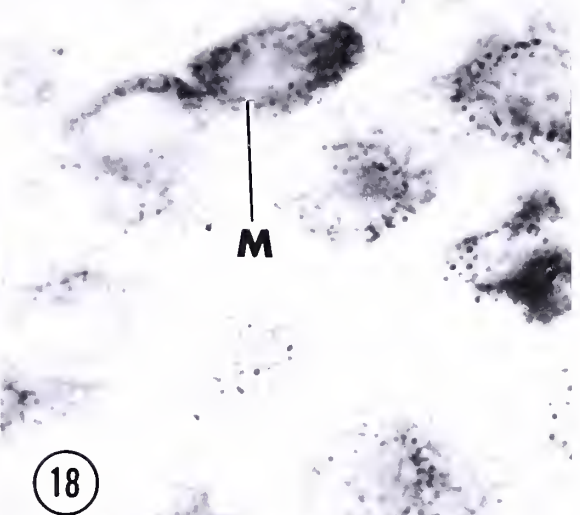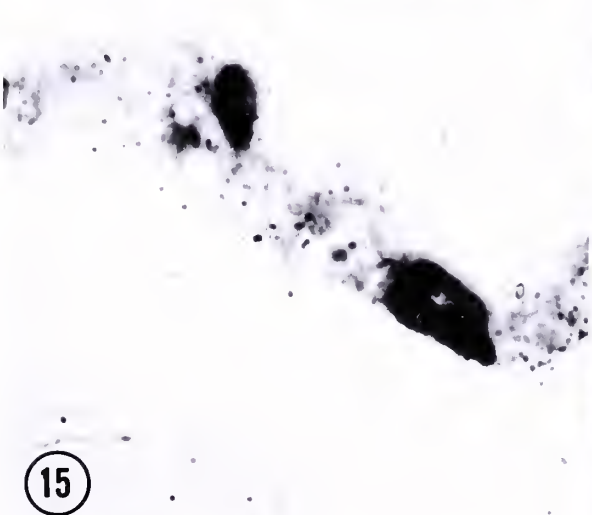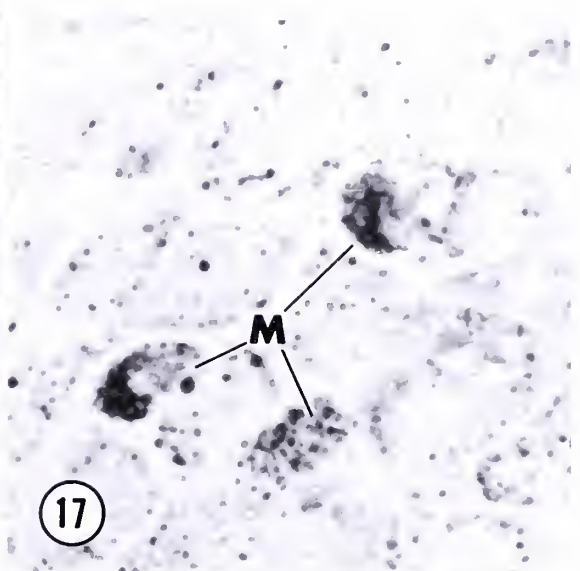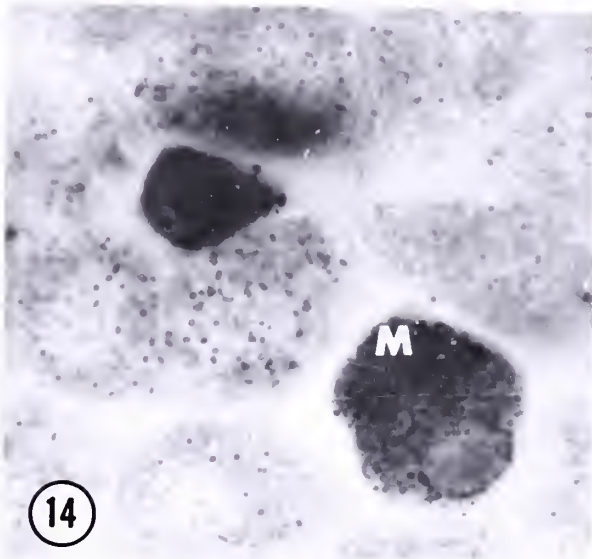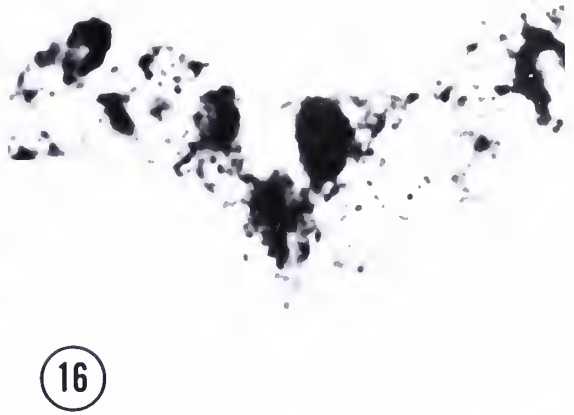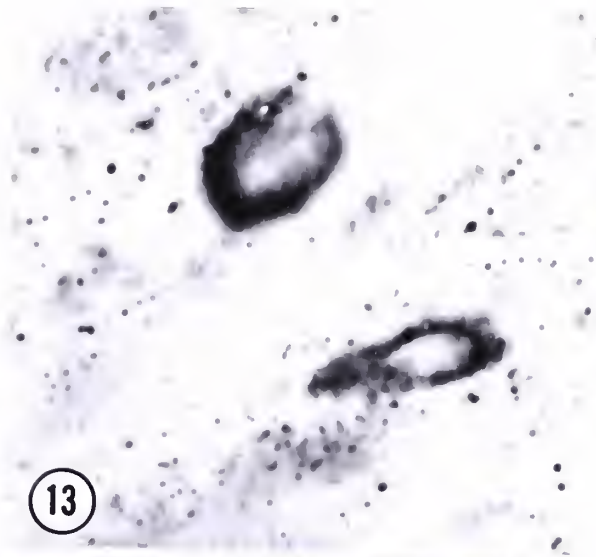
EXPLANATION OF FIGURES

Plate IV

Figure 19. G6PD, control, frozen section. Reaction product is localized
to cytoplasmic granules. Intensely reactive cells are displayed in close
proximity suggesting the distribution seen in the whole mount preparation.
(X 1250)

Figure 20. G6PD, in vitro aldosterone, frozen section. The number of
reactive granules is moderately increased over control tissue. (X 1250)

Figure 21. G6PD, in vivo aldosterone, frozen section. In contrast to
control tissue, there are many more highly reactive cells (compare with
fig. 24). (X 1560)

Figure 22. G6PD, control, whole mount. The reaction is localized to
cytoplasmic granules, with clear separation of the more reactive from
the less reactive cells. This preparation also shows the contiguous
arrangement of the highly reactive cells in a small linear cluster.(X 2300)

Figure 23. G6PD, in vitro aldosterone, whole mount. While the dis-
tibution of the reactive cells remains approximately the same as in
control tissue, the density of the intracytoplasmic granules appears
greater. (X 2300)

Figure 24. G6PD, in vivo aldosterone, whole mount. In comparison to
control tissue, the reactive cells are more numerous and arranged in
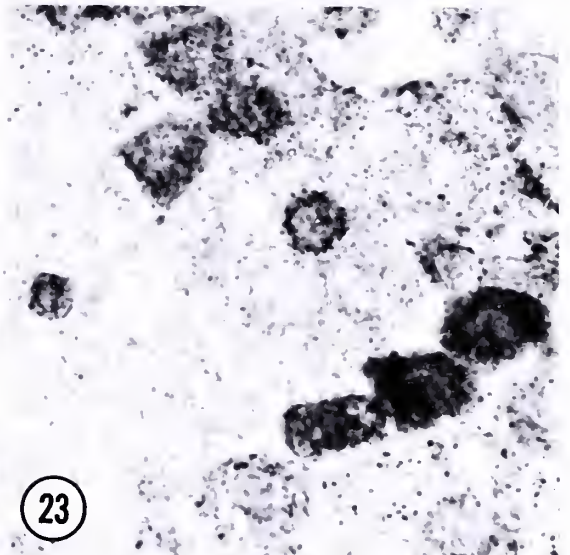larger stellate groups. (X 2300)

-48-
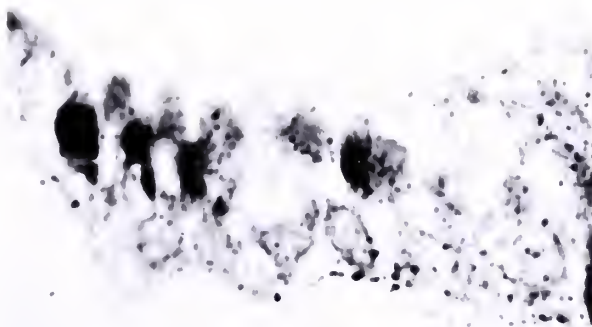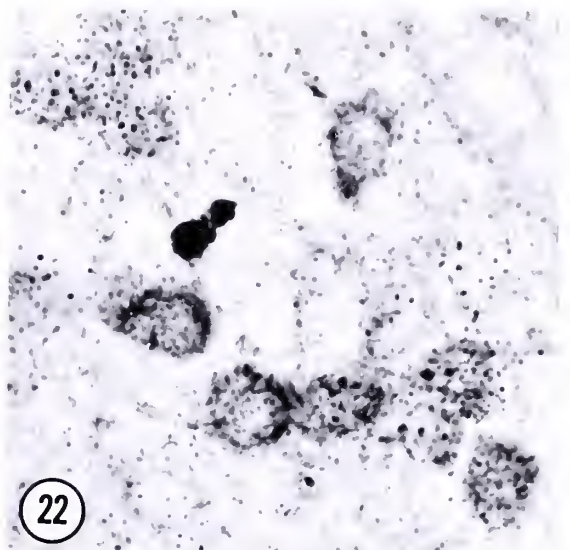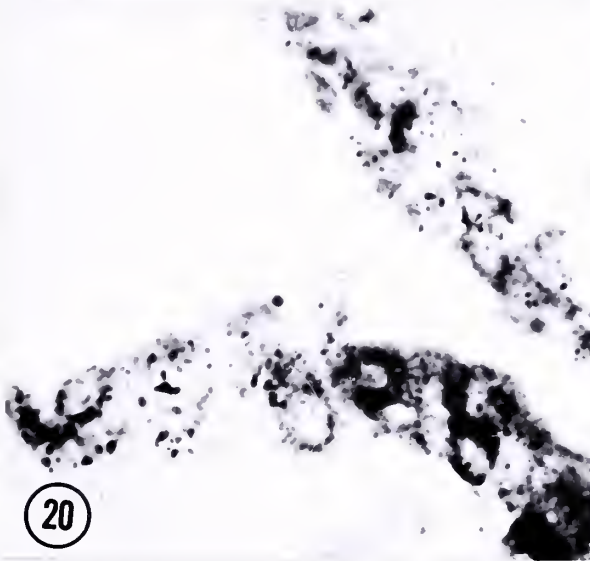
EXPLANATION OF FIGURES

Plate   V

Figure 25. ATPase, control, frozen section. Reaction is most intense
within two cells (arrow) containing enzymatic activity localized to the
cytoplasm and to granules. (X 2500)

Figure 26. ATPase, in vivo aldosterone, frozen section. Both the cyto-
plasmic and granular activity is more intense than in control tissue,
especially adjacent to the cell membrane. (X 2500)

Figure 27. G6Pase, control, frozen section. Enzyme activity is local-
ized largely to perinuclear granules of the epithelial cells. (X 2000)

Figure 28. G6Pase, in vivo aldosterone, frozen section. While cytoplasmic
localization is similar to preceding control tissue, a moderate increase
in perinuclear granules can be seen. (X 2110)

Figure 29. Non-specific esterase, control, whole mount. A few cells
are filled with reactive droplets, while the others show a vacuolar
localization adjacent to the cell membrane. (X 1875)

Figure 30. Non-specific esterase, in vivo aldosterone, whole mount.
The number of heavily reactive cells is greater than in control tissue.
Moreover, staining of the remaining cells, as indicated by the number
of vacuoles at the periphery of the cytoplasm, is more intense. (X 1875)

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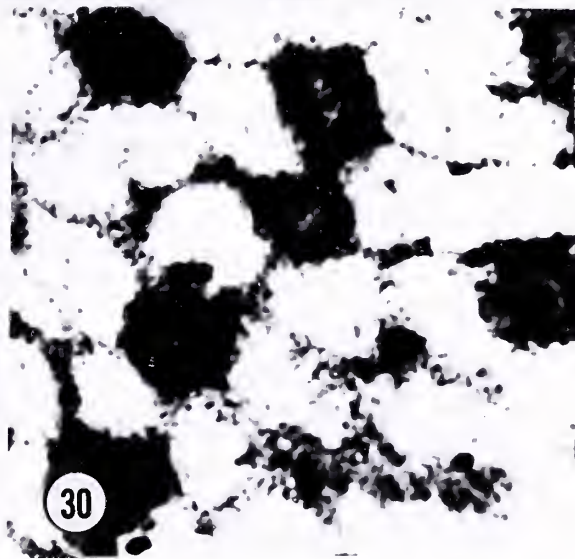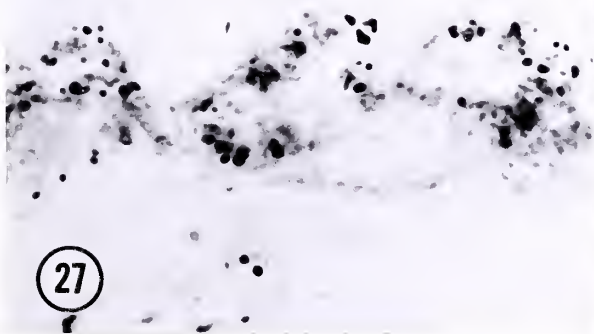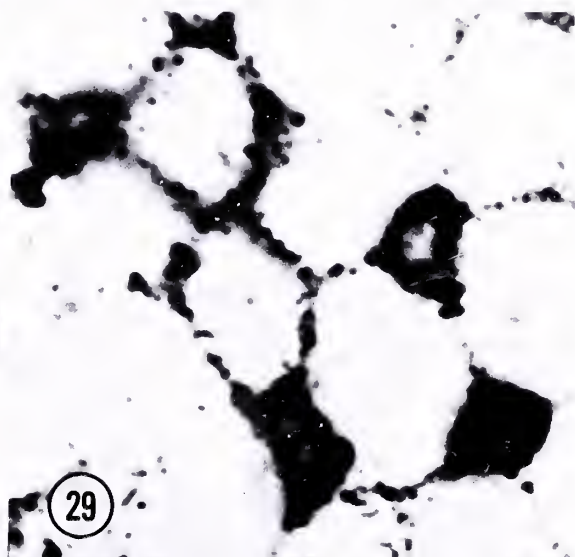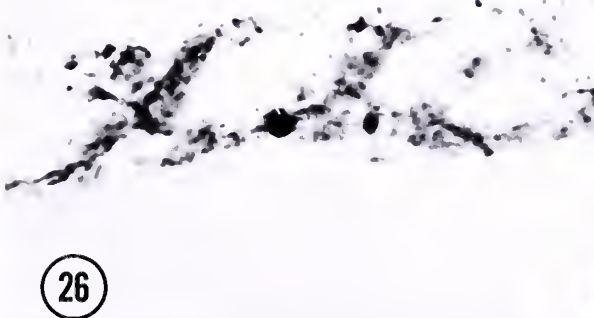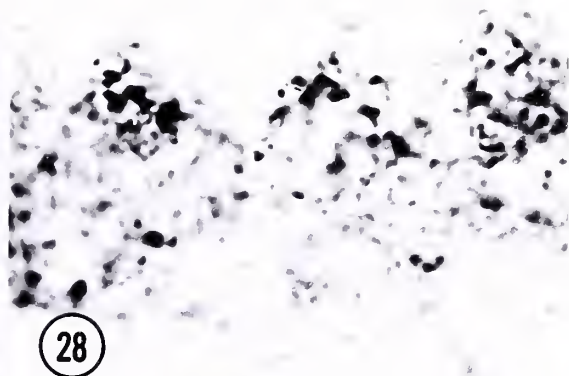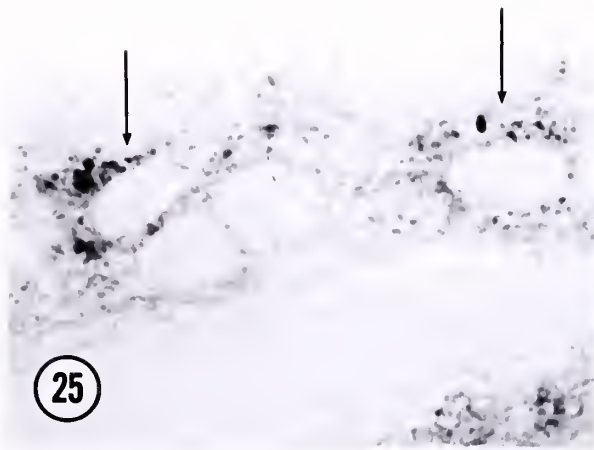
EXPLANATION OF FIGURE

Figure 31. This figure is a copy of a polarographic tracing of oxygen

uptake in mitochondria isolated from control toad bladders. Oxygen

uptake is measured along the abscissa; the ordinate represents time.

The effect of the addition of substrate and ADP can be seen. A line

is extended along each straight segment of the curve; the intersection

of each line represents a point on the abscissa from which the amount

of oxygen is determined. In this case, $P/O = \dfrac{460}{165} = 2.89$.

The ratio of the rate of oxygen consumption in States III and IV is
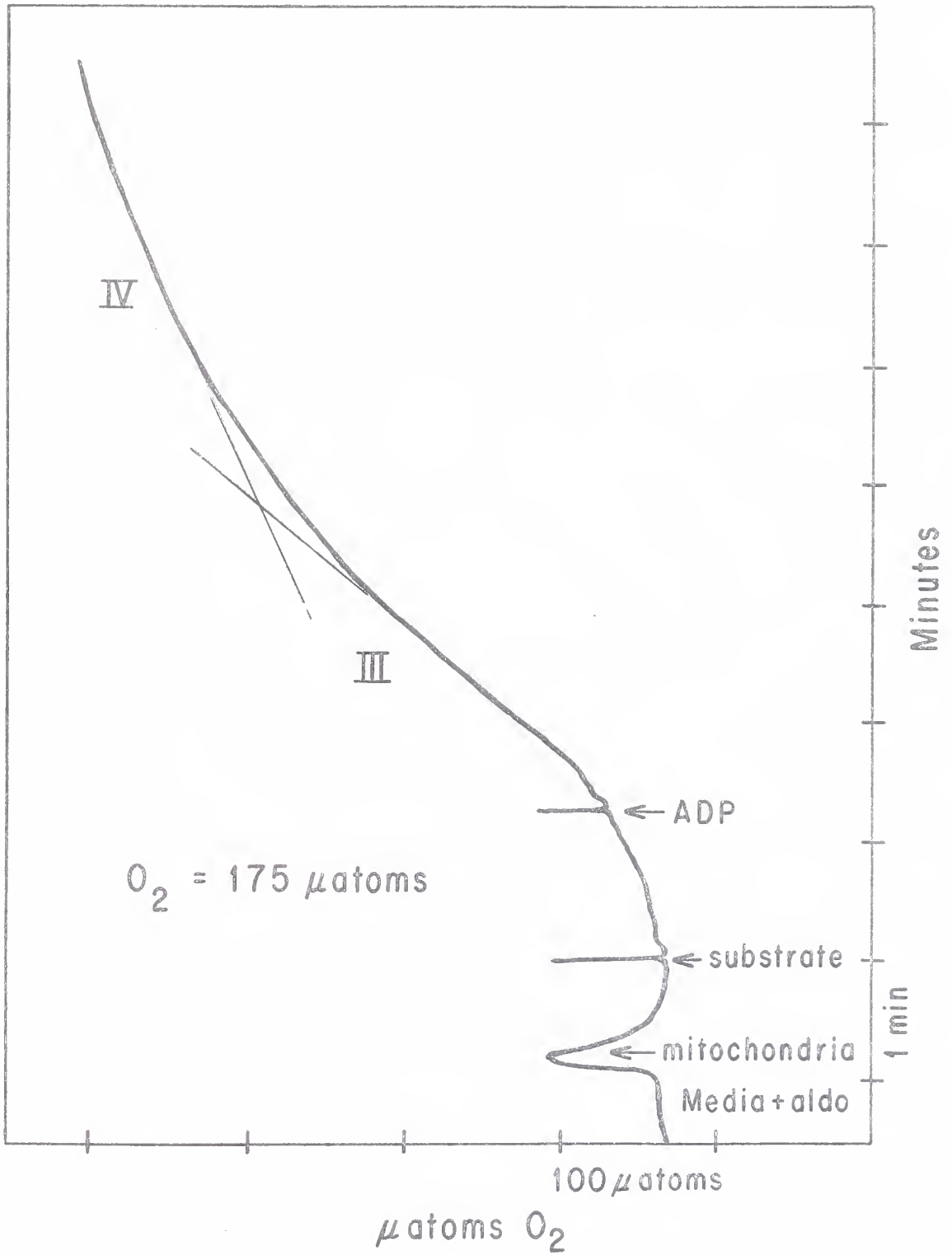
$5/.9 = 6$.

Fig. 31
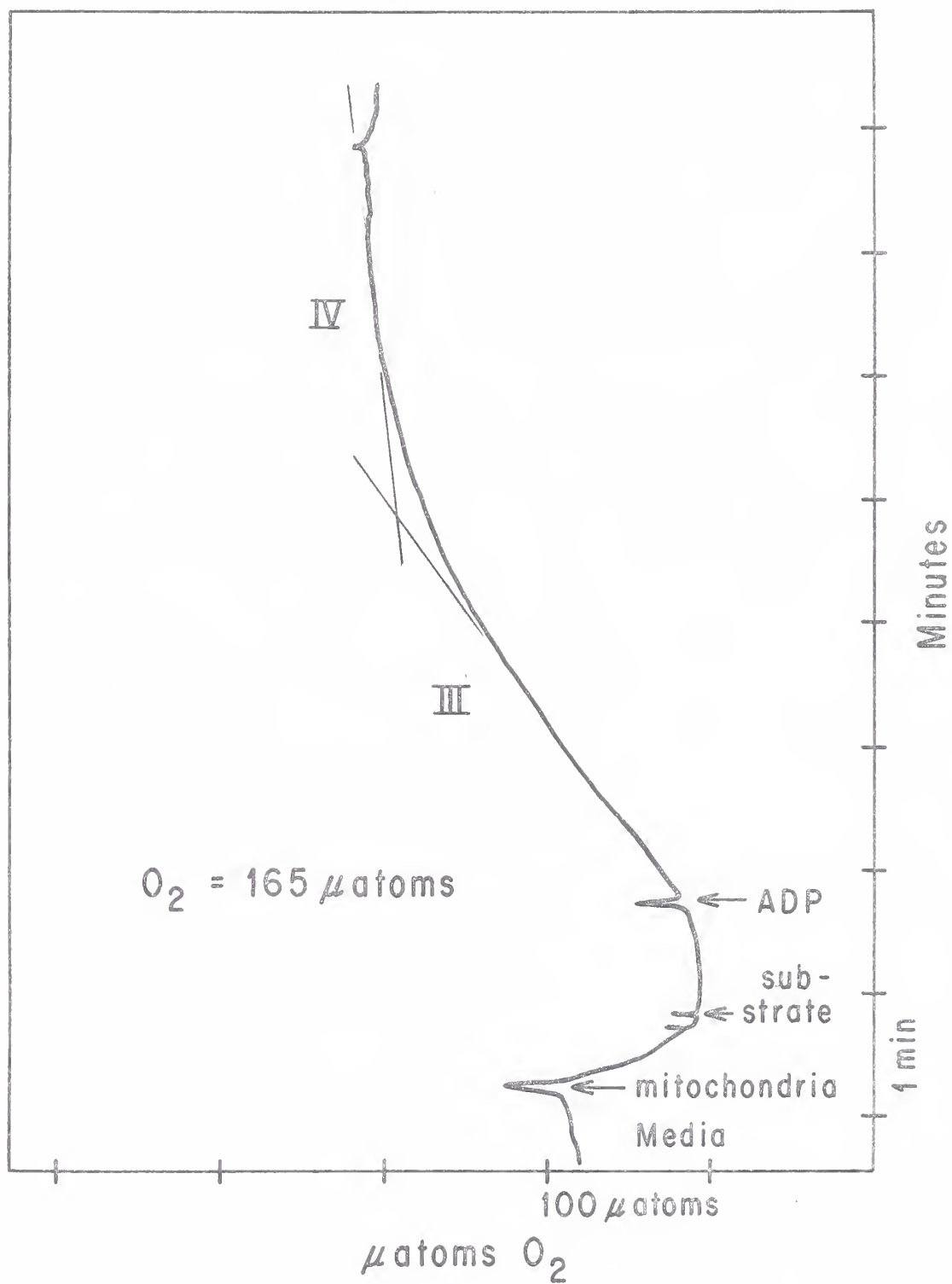
EXPLANATION OF FIGURE

Figure 32. This figure is a copy of a polarographic tracing of oxygen

uptake in mitochondria isolated from control toad bladders in which the

polarographic medium contained 10^{-7} M aldosterone. Oxygen uptake is

measured as in fig. 31. Here, P/O $= \dfrac{460}{175} = 2.61$. State III/State IV =

6/5 = 1.1. The amount and rate of oxygen consumption is greater than

that of control mitochondria.

Fig. 32

EXPLANATION OF FIGURE

Figure 33. This figure is a copy of a polarographic tracing of oxygen uptake in mitochondria isolated from toad bladders incubated in vitro with 10^{-7} M aldosterone for three hours. Oxygen consumption is measured as in fig. 31. Here, P/O = $\frac{460}{270}$ = 1.71. State III/ State IV= 8/2.1 = 4. The amount and rate of oxygen consumption is greater than both control mitochondria and mitochondria exposed to the hormone for a short period.

Fig. 33
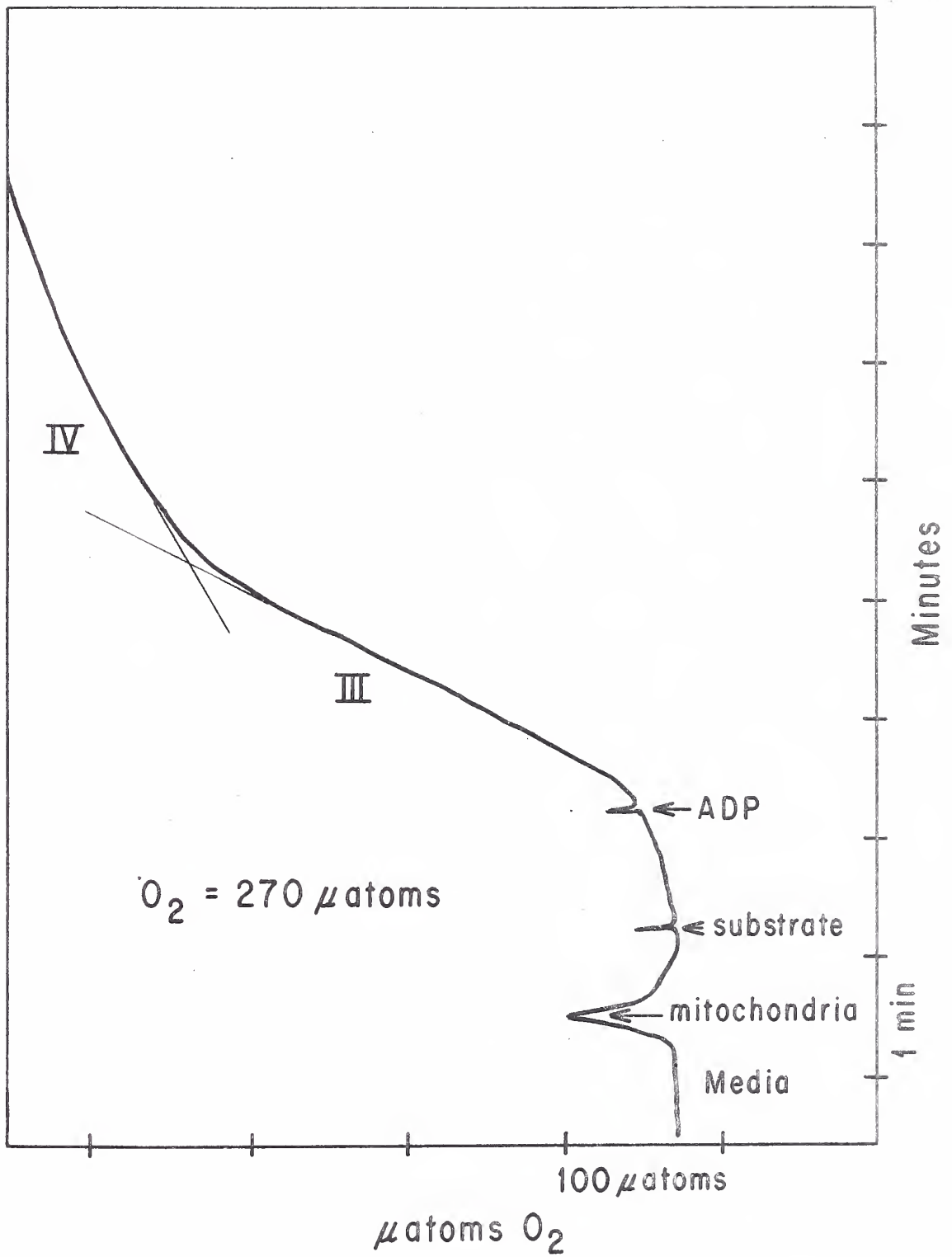
EXPLANATION OF FIGURE

Figure 34. This graph illustrates the degree of increase in short circuit current exhibited by the hemibladder exposed to 10^{-7} M aldosterone at the serosal aspect of the membrane. The control hemibladder showed no increase in short circuit current. This demonstrates the potency of the hormone preparation.

Fig. 34